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QUANTIFICATION OF THE ANALYTICAL PARAMETERS AND UNCERTAINTY BUDGET FOR TRACEABLE MEASUREMENTS OF BENZOIC ACID IN SOFT DRINKS BY HPLC METHOD

Maria Mădălina JURCOVAN [1], Elena DIACU[2], Cornelia PETRONELA ENE[3]

S-a realizat evaluarea parametrilor de performanța analitică şi a bugetului de incertitudine pentru metoda HPLC în determinarea trasabilă a acidului benzoic din băuturi răcoritoare. S-au determinat parametrii de performanță analitica: linearitatea curbei de calibrare, limita de detecție (LOD), limita de cuantificare (LOQ), gradul de recuperare, repetabilitatea, reproductibilitatea, robustețea şi stabilitatea. Toate valorile parametrilor de performanță susțin valabilitatea metodei HPLC pentru determinarea conservantului anti-microbian acid benzoic din băuturi răcoritoare si trasabilitatea acestei metode.

The evaluation of analytical performance parameters and uncertainty budget of the HPLC method for traceable determination of benzoic acid in soft drinks has been performed. This contribution reports the results obtained for the following characteristic performance parameters: linearity of calibration curve, detection limit (LOD), quantification limit (LOQ), percent recovery, repeatability, reproducibility, ruggedness and stability. The uncertainty budget of the quantitative measurement of the benzoic acid was also determined. All the values for the performance parameters argue the validity of the developed HPLC method for the determination of antimicrobial preservative-benzoic acid in soft drinks and the traceability of this method.

Key words: analytical parameters, HPLC, benzoic acid, uncertainty, soft drinks.

1. Introduction

In order to use the food that is not fresh, without both chemical and microbiological spoilage, people have used preservation methods from the beginning. In order to fulfill the consumers' desire for more variety of food items and their availability all year round, the food industry has been used over time

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[1] PhD Student, Department of Analytical Chemistry, University POLITEHNICA of Bucharest, Romania, corneliaene2007@yahoo.com

[2] Professor, Department of Analytical Chemistry, University POLITEHNICA of Bucharest, Romania, elena_diacu@yahoo.co.uk

[3] PhD Student, Department of Analytical Chemistry, University POLITEHNICA of Bucharest, Romania, madalinajurcovan77@yahoo.com

many preservation methods. Nowadays, this aspect has become even more important, as food may be produced at one location, processed in another, and later distributed to other remote locations. In this way, the interval between food production and consumption has become much longer and preservation of food over a long period has become essential. The most effective and therefore, the most extensive conservation method applied is by far the one that uses chemical preservatives. The selection of a proper antimicrobial agent depends on the food products and the expected microorganisms, and sometimes may be difficult. It is desirable to use such an antimicrobial agent capable to inhibit a wide range of spoilage causing microorganisms. Among the chemical food preservatives, benzoic acid and its salts are commonly used in numerous foodstuffs, especially in soft drinks, being a favorite choice due to their excellent preservative qualities.

Following the interest of the food industry for the preservative benzoic acid use in food processing, and of the European Union regarding the risk for consumers when food additives other than colours and sweeteners are used [1], a simple, accurate and rather fast HPLC method has been developed for identification and determination of benzoic acid in soft drinks [2]. This method consists in the direct injection of the soft drink sample in a HPLC system with UV diode array detection on a reverse phase column. This process has been described in detail in our previous work [2].

The present work describes the evaluation of analytical parameters of the HPLC method for benzoic acid determination from commercial samples of soft drink in order to validate and accredit the method. The maximum accepted level in beverages by European law is 150 mg/L [2].

The following performance analytical criteria of HPLC method have been evaluated: linearity of calibration curve, detection limit (LOD), quantification limit (LOQ), percent recovery, repeatability, reproducibility, ruggedness, stability, and measurement uncertainty, according to [3-7]. The method has been also used in international inter-laboratory testes and for soft drink market monitoring.

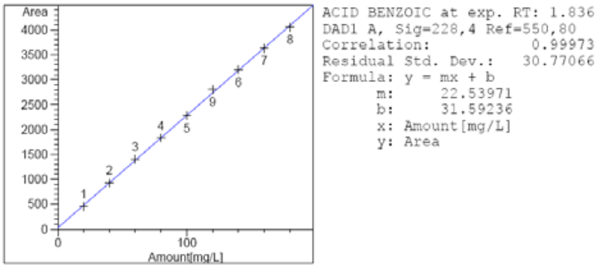
2. Experimental

All reagents used were of chromatographic purity grade and all solutions were prepared using bidistilled water. The samples were filtered on a microfiltration membrane, placed on an ultrasonic bath, and then adjusted to the pH 4.2. The chromatographic determinations were carried out using a high performance Agilent Series 1100 liquid chromatograph system with diode array detection at the wavelength of 228 nm. The separation was achieved using a mobile phase which consists in two components: acetonitrile and ammonium acetate solution.

3. Results and discussion

Calibration curve linearity

The linearity of the method was tested on the basis of peak areas of various concentrations of standard solutions of benzoic acid (BA). The working standard solutions were daily prepared from a stock solution of 1000 mg/L. The linearity of the calibration curve was registered between 0-150 mg/L, with a good regression coefficient, **R=0.999** (Fig. 1).



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ACID BENZOIC at exp. RT: 1.836
DAD1 A, Sig=228,4 Ref=550,80
Correlation:             0.99973
Residual Std. Dev.:     30.77066
Formula: y = mx + b
       m:       22.53971
       b:       31.59236
       x: Amount[mg/L]
       y: Area
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Fig. 1 Calibration curve for benzoic acid

Detection limit (LOD) and quantification limit (LOQ)

For the HPLC method, the LOD is considered as three times the standard deviation of the mean of the blank determinations, and LOQ is three times LOD. As the analytical signal is zero for the blank sample, LOD can be considered as the concentration at wich the ratio *signal/noise* is 3/1. In order to establish the LOD, different concentrations of benzoic acid standard solutions were analysed. The results for the different concentrations are presented in Table 1 and show a LOD equal with 7 mg/L. Consequently, the LOQ is 21 mg/L.

Table 1

Determination of LOD

BA concentration (μg/L)	2,5	5	7	10
Signal/noise	1,6	2,4	2,9	3,8

Percent recovery

In order to determine the percent recovery of the benzoic acid analyse by HPLC, a standard soft drink was analyzed (unfortified sample). No analytical signal was generated in this case, so the preservative content is considered to be 0 mg/L. Taking into account the maximum limit acceptable by law, a set of 18 samples of soft drink were fortified at three concentration levels, 6 samples for each level of concentration, as displayed in Table 2, where C_d represents the determined concentration and C_I is the concentration of the blank. It can be seen that the recovery percentage for benzoic acid is situated between 85.69% and 92.86%.

Table 2

First experimental data for determination of the percent recovery

100 mg/L fortification level						
C_D-C_I, mg/L	92,52	92,86	92,64	92,53	89,47	89,25
% recovery	92,52	92,86	92,64	92,53	89,47	89,25
150 mg/L fortification level						
C_D-C_I, mg/L	132,29	132,32	132,33	132,351	132,74	132,05
% recovery	88,19	88,21	88,22	88,23	88,49	88,03
200 mg/L fortification level						
C_D-C_I, mg/L	171,399	171,59	171,59	171,46	171,59	172,08
% recovery	85,69	85,79	85,79	85,73	85,79	86,04

Repeatability

Repeatability (r) is the value below which the absolute difference between single test results obtained under repeated conditions (i.e., same sample, same operator, same apparatus, same laboratory, and short interval of time) may range within a specific probability (typically 95 %). It results r = 2.8 × s_r. (s_r - standard deviation). As in the previous work the same HPLC instrument it has been used, operated by the same analyst, in the same day, the respective values for C_D-C_I being applied in order to establish the repeatability. The obtained values for s_r (standard deviation of the repeatability r), and *cv* (variation coefficient) for benzoic acid are presented.

Table 3

Determination of repeatability for benzoic acid analyse

No. crt.	Parameter	Benzoic acid, mg/L		
		100	150	200
1.	s_r	1.7	0.2	0.2
2.	r	4.7	0.6	0.6
3.	*cv* %	1.5 mg/L	0.13 mg/L	0.1 mg/L

Reproducibility

In order to verify the reproducibility (**R**), two sets of experiments have been performed in two different days. First set of experiments, collected within 1 day are those presented in Table 2. In Table 4 are the analytical data obtained in the same conditions as those from Table 2, but in a different day. From the displayed data, it can be seen that for all three fortification levels of benzoic acid, the values of s_R is smaller than 2, fact which gives reproducibility to the proposed method.

Table 4

Second experimental data for determination of the percent recovery for the determination of reproducibility and mean results for s_R[*], r[**], and *cv*

No. crt.	Analyte / Parameter	Samples of benzoic acid in the second experiment					
1.	Fortified level	100 mg/L					
2.	C_D-C_I, mg/L	52.03	52.61	52.44	52.60	52.40	52.10
Mean results		**$s_r = 0.260$**		**r = 0.73**		***cv*% = 0.49**	
3.	Fortified level	150 mg/L					
4.	C_D-C_I, mg/L	102.18	102.10	101.98	102.00	101.94	102.07
Mean results		**$s_r = 0.088$**		**r = 0.25**		***cv*% = 0.08**	
5	Fortified level	200 mg/L					
6.	C_D-C_I, mg/L	148.49	148.48	148.49	148.60	148.46	148.51
Mean results		**$s_r = 0.050$**		**r = 0.14**		***cv*% = 0.03**	
		$s_{R\ (50)} = 0.17$		**$s_{R\ (100)} = 0.11$**		**$s_{R\ (150)} = 0.05$**	

[*] $s_R = (s_{r,\text{ first experiment}} + s_{r,\text{ second experiment}})/2$; [**] $r = (r_{\text{first experiment}} + r_{\text{second experiment}})/2$.

Ruggedness/Stability

The ruggedness of the method was tested, checking if the requirement $s_D > s_R$ for HPLC method is fulfilled. One soft drink sample was fortified at 100 mg/L concentration level of benzoic acid, and it was analysed mainly using the same instrumental operating parameters as indicated in the method for benzoic acid determination, but with minor changes. These consist in a slightly different pH value for the mobile phase, and elution gradient ratio. Therefore, the pH for the component B was changed to 3.8, in comparison with the mentioned value of 4.2, corresponding of the developed method [2]. The elution gradient ratio was A/B=45/55 instead of a 40/60 used in [2]. In both cases, the analytical peak is shifted out of accepted limits, and the benzoic acid cannot be identified and analysed under these parameters. Accordingly, the method has not ruggedness under the chosen modifications. Table 5 reports the results of the retention times for benzoic acid.

Table 5

Retention times (Rt) for benzoic acid for two samples at pH=3.8 and pH=4.2

No. Crt.	Modified parameters		R_t in [2] (min)	R_t in the present work (min)
	pH	Gradient		
1.	3.8	A/B=40/60	1.72	2.5
2.	4.2	A/B=45/55	1.72	1.2

The stability of the method was checked by drawing the control map (Fig. 2). This map presents in Y-axis the characteristics of 20 individual sample points values, plotted on the X-axis: the percent recovery, the average of the individual recovery values, the average of the individual recovery values ± 2xSD and the average of the individual recovery values ± 3xSD.

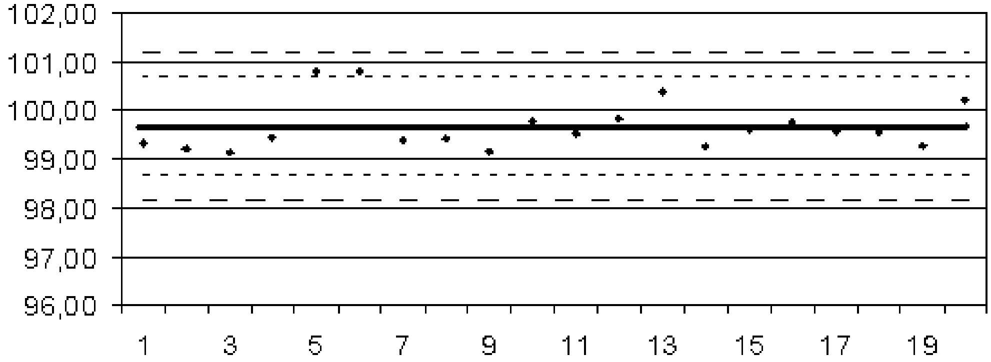

Fig. 2. Control-map stability for benzoic acid: individual percent recovery values (♦); average of percent recovery values (▬); average of percent recovery values ± 2 x STD (··); average of percent recovery values ± 3 x STD (– –).

Measurement uncertainty

The following individual uncertainty components of the total measurement uncertainty in the analysis of benzoic acid in soft drinks by HPLC method can be identified: 1- preparation of working standard solution of 100 mg/L; 2-percent recovery; 3- reproducibility; 4- associated uncertainty for HPLC instrument.

1. Associated uncertainty on working solution preparation

1.1. Uncertainty on stock solutions preparation

Two contributions to the uncertainty on stock solutions preparation have to be taken into account: weighing at analytical balance and measuring the volume in 100 mL volumetric flask.

To prepare 100 mL of the stock solution of 1000 mg/L, 100 mg of benzoic acid is weighted using an analytical balance. The calibration certificate of Mettler Toledo AT 261 analytical balance has an accuracy of ± 0.01 mg for 100 mg, an uncertainty, u = 0.005 mg, with a coverage factor k = 2. In order to establish the contribution of weighing on an analytical balance to the uncertainty on stock solutions preparation, 10 replicate weighing were performed, as presented in Table 6.

Table 6

Replicate weighing for the preparation of benzoic acid stock solutions

Replicate number	Benzoic acid (mg)
1	100.5
2	100.2
3	100.0
4	100.3
5	1002
6	1004
7	0.1001
8	0.1003
9	0.1005
10	0.1004
Average	0.10029
SD	0.000166

The uncertainty of stock solution preparation, $u(m_{stoc})$ is given by:

$$u(m_{stoc}) = \sqrt{(0{,}005)^2 + (0{,}0001655)^2} = 0{,}0050$$

The uncertainty of a 100 mL volumetric flask Class A can have three influences on the uncertainty on stock solutions: the measuring flask error, the repeatability error and the temperature effect error.

The contribution of the A class 100 mL measuring flask (Hirschmann Laborgeräte GmbH & Co - Germany) to the volume measurement uncertainty is calculated from the tolerance ± 0.1 at 20^o C temperature (provided by the producer), considering a rectangular distribution:

$$u(V_{Flask\text{-}100mL}) = 0.1/3^{1/2} = 0.0577 \text{ mL}$$

The repeatability uncertainty results from the standard deviations of 10 replicate fillings and weightings of the 100 mL volumetric flask is: $u(V_{repeatability\ -100mL})$ = 0.0261 mL.

The contribution to the uncertainty of the flask calibration temperature (20^o C) is assessed taking into account the temperature variation limits of ±4˚C and using the volume expansion coefficient. For the aqueous solution this coefficient is $2.1 \bullet 10^{-4}$ and the dilatation coefficient with the temperature is $100 \times 4 \times 2.1 \times 10^{-4} = 0.084$. The uncertainty due to variation of the temperature, assuming a rectangular distribution is:

$$u(V_{\text{Temp.variation 100mL}}) = \frac{0.084}{\sqrt{3}} = 0.0485 \text{ mL}.$$

The three uncertainty contributions make the combined uncertainty for 100 mL volumetric flask:

$$u(V_{\text{stoc}}) = \sqrt{(0.0577)^2 + (0.0261)^2 + (0.0485)^2} = 0.0797 \text{ mL}$$

The quantification of all uncertainty contributions on stock solutions preparation is given below and the known formula for the combined uncertainty is applied:

$$\left(\frac{u(c_{stoc})}{c_{stoc}}\right) = \sqrt{\left(\frac{u(m)}{m}\right)^2 + \left(\frac{u(V)}{V}\right)^2 + \left(\frac{u(P)}{P}\right)^2}$$

$u(P)=0.0005/\sqrt{3}= 0.000289$ (Table 7).

Table 7

Uncertainty's contributions for the stoc solution

Parameter	x	Uncertainty, u(x)	u(x)/x
m	100 mg	0.0052	0.000052
V	100 mL	0.0797	0.000797
P	0.995	0.000289	0.000003

$$\frac{u(c_{stoc})}{c_{stoc}} = 0{,}00079859$$

The combined uncertainty for 1000 mg/L benzoic acid stock solution, $u(c_{\text{stoc}}) = 0{,}00079859 \cdot 1000 = 0{,}7986$ mg/L (ppm), where c is the concentration of stock solution.

1.2. Uncertainty on working solution preparation

Two contributions to the uncertainty have here to be taken into account: 10 mL volumetric flask and micropipette. To prepare 100 mL of working solution of stock solution 100 mg/L, 1 mL of stock solution 1000 mg/L is diluted in a volumetric flask of 10 mL. In order to establish the contribution of the measurement of the volumes on the uncertainty on working solutions similar procedure and calculus are performed as for the uncertainty on stock solutions preparation.

The contribution of the A class 10 mL measuring flask (Hirschmann Laborgeräte GmbH & Co - Germany) to the uncertainty on the volume measurement is calculated from the tolerance ± 0.025 at 20^{o} C temperature (provided by the producer), considering a triangular distribution:

$$u(V_{Flask\text{-}10mL}) = \frac{0.025}{\sqrt{6}} = 0.0102 \text{ mL}$$

The second contribution to this uncertainty is the repeatability. According with the Class A 10 mL volumetric flask certificate, the standard deviation is 0.006 mL, taken as uncertainty it is:

$u(V_{repeatability\ -10mL}) = 0.006$ mL.

The uncertainty of the flask calibration temperature is calculated from the dilatation coefficient with the temperature ($0.008 = 10 \times 4 \times 2.1 \times 10^{-4}$) , using a rectangular distribution for the volumetric flask of 10 l mL:

$u(V_{temp\ variation\ 10mL}) = 0.008/3^{1/2} = 0.0046$ mL

All three calculated contributions make the combined incertitude for the 10 mL volumetric flask uncertainty:

$$u(V_{lucru}) = \sqrt{(0.0102)^2 + (0.0060)^2 + (0.0046)^2} = 0.0127 \text{ mL.}$$

To measure 1 mL stock solution a micropipette of 100-1000 μL is used. There here Two contributions are possible: the uncertainty due to the micropipette itself and to the micropipette repeatability. The contribution of the micropipette (BRAND, Germany) to the uncertainty on 1 mL measurement is assed from the indicated value of the micropipette certificate, which is 0.0015 mL. Considering that it represents a normal distribution we get:

$u(V\mu_{Pipette\text{-}100mL}) = 0.0015/2 = 0.00075$ mL

The repeatability of the micropipette is obtained from the standard deviation provided by the producer, considering a rectangular distribution:

$u(V_{repeatability\ -\mu Pipette}) = 0.003/3^{1/2} = 0.00173$ mL

The sum of two contributions give the associated uncertainty of the micropipette is:

$$u(V_{\mu p}) = \sqrt{(0.00075)^2 + (0.00173)^2} = 0.0019 \text{ mL.}$$

To calculate the associated uncertainty on working solution preparation, denoted with $u(c_{working})$, the bellow disposal of all contributions is taken into account and the known formula is applied:

$$\left(\frac{u(c_{working})}{c_{working}}\right) = \sqrt{\left(\frac{u(c_{stock})}{c_{stock}}\right)^2 + \left(\frac{u(V_{stock})}{V_{stock}}\right)^2 + \left(\frac{u(V_{working})}{V_{working}}\right)^2 + \left(\frac{u(V\mu p)}{V\mu p}\right)^2}$$

Table 8

Uncertainty's contributions for the working solution

Parameter, x	Uncertainty u(x)	u(x)/x
c_{stoc}, 1000 mg/L	0.7986	0.0007986
V_{stoc}, 100 mL	0.0797	0.000797
$V_{\mu p}$, 1 mL	0.0019	0.0019
$V_{working\ sol}$, 10 mL	0.013	0.0013

$u(c_{working}) = 0.00254 \cdot 100 = 0.254$ mg/L

2. Uncertainty related to the recovery and to the reproducibility

The uncertainty related to the recovery was calculated by the integration of relative standard deviations for benzoic acid, $RSD_{benzoic\ acid}$, which being considered as the uncertainty related to the recovery within 1 day. The analytical data for level concentration 150 mg/L of standard deviation (SD) and relative standard deviation (RSD) of recovery percentage fall between 88.03-88.49 %, with an average of 88.23. The associated recovery uncertainty is the RSDs sum of the values within 1 day, amount to 0,0017.

The reproducibility was verified by calculating the RSDs values of six time analyses of a fortified soft drink sample at 150 mg/L level for benzoic acid, performed between days. Results are reported in Table 9. $RSD_{benzoic\ acid} = 0.0013$.

Table 9

Uncertainty related to the reproducibility

Recovery one day %	132.29	132.32	132.33	132.35	132.74	132.05
Recovery different day %	132.26	132.34	132.28	132.35	132.59	132.4
Average	132.35	132.37				
SD	0.222	0.11837				
RSD	0.0017	0.0009				
Uncertainty	0.0013					

3. Associated uncertainty for HPLC instrument

The associated uncertainty for HPLC instrument $u_{instrument\text{-}HPLC}$ was determined, accordling with Table 10.

Table 10

Analytical data for associated uncertainty for HPLC

Conc. mg/L)	Area	
10.000	212.22	Uncertainty = **2,23 mg/L**
20.000	449.93	
50.000	1124.55	
80.000	1757.30	
100.000	2240.39	
120.000	2733.38	
150.000	3282.307	

Finally, the measurement uncertainty, denoted U, can be expressed either as the *"combined standard uncertainty"* of the results, denoted with u, or as *"expanded uncertainty*", symbolised with U.

Combining the uncertainty components, u is obtained:

u = 1.13 mg/L

The expanded uncertainty **U** is calculated using the simple relationship **U =** *u* • k, where k is the coverage factor. Considering a normal distribution, k=2, U = 2.26 mg/L.

4. Conclusions

The analysis of analytical parameters of the HPLC method for benzoic acid determination in soft drinks has validated the high performance of the method, making possible its accreditation by the national accreditation body. As it has been shown, all the studied parameters satisfy the requirements of metrological standards.

The influence of the best results obtained for the method performance parameters are highlighted in the excellent value of measurement uncertainty of 1.13 mg/L benzoic acid, which is appreciated for HPLC method. Following participation in international tests to determine benzoic acid in soft drinks, the method has achieved a very good score, and therefore is considered traceable and competitive. The method also complies with the European regulations, being a reliable method for monitoring of the quality of the beverage market regarding the compliance with the legal limit (150 mg/L), since now the use of benzoic acid as a food additive is permitted in many UE countries.

R E F E R E N C E S

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